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ABSTRACT

SYNUCLEIN γ (SNCG) AND BREAST CANCER PROGRESSION.

There continues to be uncertainty as to whether premalignant lesions that ultimately develop into metastatic breast cancer can be identified histologically. Many of the genetic changes during malignant progression manifest them as alterations in the cellular complement of novel transcribed mRNAs. We have previously reported the isolation of a new breast cancer specific gene BCSG1 by differential cDNA sequencing. BCSG1, which was high abundance in a breast cancer cDNA library but scarcely in a normal breast cDNA library, was identified as a putative breast cancer marker. Interestingly, BCSG1 revealed no homology to any other known growth factors or oncogenes; rather BCSG1 has a great sequence homology with Alzheimer disease (AD)-related neural protein synuclein (SNC) that are mainly expressed in brain, and thus was also named as synuclein γ (SNCG). The SNCs have primarily been studied as neural proteins, as they are highly expressed in neurons and appear to be involved in the etiology of neurodegenerative diseases. We demonstrated that 1) SNCG expression is a stage-specific in human breast: undetectable in normal or benign breast lesions, low level and partial expression in low grade ductal carcinoma *in situ* but extremely high level in advanced infiltrating breast cancer; 2) overexpression of SNCG in human breast cancer cells leads to a significant increase in motility and invasiveness in vitro and a profound augmentation of metastasis; and 3) expression of SNCG in breast cancer cells is significantly down-regulated by growth inhibitory cytokine oncostatin M (OM). The knowledge gained from these studies will allow us to target specific pathway contributory to the breast cancer progression. In addition, if overexpression provides a therapeutic target, then BCSG1 may be useful in clinical management and treatment of breast cancer.

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I. BACKGROUND AND SIGNIFICANCE

I-1. Identification of BCSG1.

We undertook a search, using the differential cDNA sequencing approach as we previously described (1-3), for isolation of differentially expressed genes in the cDNA libraries from normal breast and breast carcinoma. Of many putative differentially expressed genes, a breast cancer specific gene, BCSG1, which was (a) highly expressed in mammary gland relative to other organs and was (b) high abundance in a breast cancer cDNA library but scarcely in a normal breast cDNA library, was identified as a putative breast cancer marker (1). The BCSG1 gene is transcribed into a 1 kb mRNA, and encodes a 127-amino acid polypeptide. Comparison of the predicted amino acid sequence with genetic databases reveals that BCSG1 is highly homologous to a family of neuronal cytosolic proteins, namely synucleins that are mainly expressed in brain and are localized to presynaptic terminals (4-6). Subsequent to the isolation of BCSG1, synuclein γ (7) and persyn (8) were independently cloned from a brain genomic library and a brain cDNA library. The sequences of these two brain proteins were found to be nearly identical to BCSG1. The five nucleotide difference found between BCSG1 and the sequences reported for synuclein γ and persyn are the results of natural nucleotide polymorphisms (8). Thus, BCSG1 is now also named synuclein γ or persyn and is considered to be the third member of the synuclein family.

I-2. Neural protein synuclein

Synucleins are a protein family consisting of 3 known members, α, β, and γ synuclein. These proteins were named synuclein based on findings indicating both synaptic and nuclear distribution. Synucleins are thought to be involved in neuronal plasticity and the formation of depositions in brain tissues (9). These proteins are predominantly present in brain. α and β synuclein are enriched at presynaptic terminals. In addition, synucleins are also present in ocular tissues, while α synuclein and β synuclein are predominantly present in the inner plexiform layer of retina, γ synuclein is in the fiber layer of optic nerve (10). Synucleins have been implicated in neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD) (11). The pathological hallmark of AD is amyloid deposition in neurotic plaques and blood vessels (12). The major constituent of amyloid is a 39-43 AA peptide named Aß component (12). A second intrinsic constituent of amyloid has been isolated and named synuclein α (SNCA) (4). Recently, the second synuclein, β synuclein, was cloned from human brain and has 61% identical sequence with SNCA (6). The previously identified BCSG1, which is also highly expressed in brain (1), shares 54% and 56% amino acid sequence identity with synuclein α and synuclein β, respectively and has been renamed as synuclein γ (SNCG) (9). Thus, the previously unrecognized homology between these proteins defines a family of human brain synucleins that currently has three members. The N-terminal halves of α synuclein, β synuclein, and γ synuclein are highly conserved. However, while the residues near the C-terminus of α synuclein are similar to those of β synuclein, those of γ synuclein diverge greatly from the α synuclein conterpart (14). Although they are homologous, each synuclein is encoded by a different gene on chromosomes 4q21.3-q22 (α synuclein), 5q35 (β synuclein), and 10q23 (γ synuclein) (14).

Synucleins have been specifically implicated in some neurodegenerative diseases particularly Alzheimer's (AD) and Parkinson's (PD) diseases. α-synuclein is a major component of Lewy bodies (LB) and Lewy neurites in sporadic PD and a subtype of AD known as LB variant of AD (11,13). LBs are also present in familial AD caused by presenilin and amyloid precurser protein gene mutations. Additionally, α-synuclein is a major component of glial cytoplasmic inclusions

(GCIs) in multiple system atrophy (MSA) as well as the neuronal inclusions and GCIs in Hallervorden -Spatz disease (11). β and γ synuclein have also been recognized to play a role in the pathogenesis of PD and LB cases (11). γ synuclein also increases the susceptibility of neurofilament-H to calcium-dependent proteases and thereby influencing **neurofilament** network integrity (15). Although their normal cellular functions are unclear several observations suggest the synucleins may serve to integrate presynaptic signaling and membrane trafficking (16). <u>Interestingly, our data indicate that the increased expression of SNCG correlates with breast progression and leads to more malignant metastatic phenotype. This is the first report indicating the potential involvement of synuclein in the non-neurotic disease.</u>

I-3. Expression of BCSG1/SNCG in breast cancer

Being identified as a breast cancer specific gene, BCSG1 mRNA was detected in neoplastic breast epithelial cells but not in normal mammary epithelial cells (1). Northern blot analysis detected a 1 kb transcript corresponding to BCSG1 mRNA in 2/4 human breast cancer cell lines derived from pleural effusions and 4/4 breast cancer cell lines derived from ductal infiltrating carcinomas (1). *In situ* hybridization analysis has demonstrated a stage-specific expression pattern of BCSG1 mRNA varying from virtually no detectable expression in normal or benign breast tissue to low level and partial expression in low grade breast carcinoma *in situ* to high expression in advanced infiltrating carcinomas (1). To confirm this stage-specific expression pattern of BCSG1, we did RT-PCR analysis of BCSG1 expression in human mammary tissues. While the expression of BCSG1 in normal breast is non-detectable (0 out of 7 normal breast specimens), 43% of stage II/III breast carcinomas (6 of 14) and 73% of stage IV breast carcinomas (11 of 15) expressed BCSG1, respectively (Section II). Western analysis to examine BCSG1 protein expression in human breast tissues showed a similar pattern in that it was not detected in normal breast tissues and stage I/II ductal breast carcinomas, but was detected in 70% of Stage III/IV ductal breast carcinomas (17). Ninkina et al were also able to confirm by using Northern and Western blotting that some breast tumors and breast tumor cell lines expressed BCSG1/SNCG, whereas normal breast tissue did not (8).

I-4. Expression of BCSG1 in other malignant tumors.

In addition to the link between BCSG1/SNCG and breast cancer progression, it has also been found that synucleins, especially γ and β synuclein, are involved in ovarian cancer. Following our identification of BCSG1, Lavedan et al first suggested that BCSG1/SNCG may be abnormally expressed in ovarian tumors as well as in breast tumors, based on the discovery of some SNCG ESTs in the libraries derived from an ovarian tumor (7). This suggestion was further confirmed by Western and immunohistochemical analyses (17). While synucleins (α, β, and γ) expression was not detectable by immunohistochemistry in normal ovarian epithelium, 87% (39 of 45) of ovarian carcinomas were found to express at least 1 type of synuclein, and 42% (19 of 45) expressed all 3 synucleins (α, β, and γ) simultaneously. Highly punctate BCSG1/SNCG expression was also observed in 20% of preneoplastic lesions of the ovary, including epithelial inclusion cysts, hyperplastic epithelium, and papillary structures, suggesting that BCSG1/SNCG up-regulation may occur early in the development of some ovarian tumors (17).

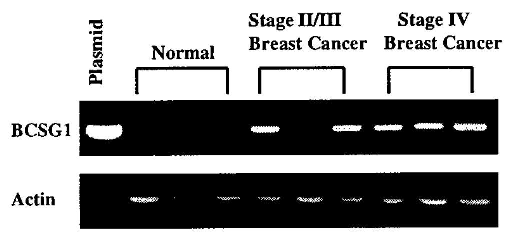
II. WORK ACCOMPLISHED. The overall hypothesis to be evaluated is that SNCG play a critical role in breast cancer malignant progression leading to metastasis. The overexpression of

BCSG1 may correlate with clinical aggressiveness of breast cancers. Therefore an alternations of BCSG1 expression may lead to an abnormal growth and malignant progression.

<u>Task 1</u>: Biological relevance of BCSG1 expression to breast cancer progression

A. Screening of BCSG1 expression in clinical breast specimens. FINISHED

Our in situ hybridization analysis has demonstrated a stage-specific expression pattern of BCSG1 mRNA varying from virtually no detectable expression in normal or benign breast tissue to low level and partial expression in low grade in situ breast carcinoma to high expression in advanced infiltrating carcinomas (1). In the grant proposal, we proposed to continue use in situ hybridization analysis of BCSG1 expression in clinical breast specimens. Because of the **non-quantitative** nature of in situ analysis, we performed a RT-PCR analysis in stead of the originally proposed in situ analysis. To verify the stage-specific expression pattern of BCSG1, we analyzed 36 clinical breast specimens including normal or benign lesions, stage II/III breast carcinomas, and stage IV breast carcinomas. As shown in Fig. 2, while no BCSG1 mRNA was detectable in 7 breast specimens of normal or benign hyperplasia, BCSG1 mRNA was expressed in 43 % (6 of 14) and 73 % (11 of 15) of stage II/III and stage IV breast carcinomas, respectively.



Expression of BCSG1/SNCG in human breast

Stage	Normal (n=7)	Stage II/III (n=14)	Stage IV (n=15)
Expression	0 (0%)	6 (43%)	11 (73%)

Fig. 1. Expression of BCSG1 in human breast tissue. Total RNA was isolated from frozen human breast specimens. RT-PCR analysis of BCSG1 using primers within BCSG1 cording sequence (Sense: 5'-ATGGATGTCTTCAAGAAGGG-3'; antisense: 5'-CTAGTCTCCCCCACTCTGGG-3'). The 384-bp PCR product is a specific indication of the presence of BCSG1. The integrity and the loading control of the RNA samples were ascertained by actin expression with a set of primers (5'-GCTGTGCTATCCCTGTACGC-3' and 5'-TGCCTCAGGGCAGCGGAACC-3') for 314-bp β-actin

Using Western blot, Godwin AK's group also demonstrated a similar BCSG1 **protein** expression pattern in human breast samples. BCSG1 protein expression was not detectable in either normal breast or ductal carcinoma in situ (0 of 3) or Stage I/II breast carcinoma (0 of 6). However, 70% (12 of 17) of Stage III/IV breast carcinomas expressed SNCG protein. To emphasize the similarity and the importance of this stage-specific BCSG1 expression in breast tissue, PI downloaded Dr. Godwin's data here as Fig. 2 on BCSG1 expression in breast tumors.

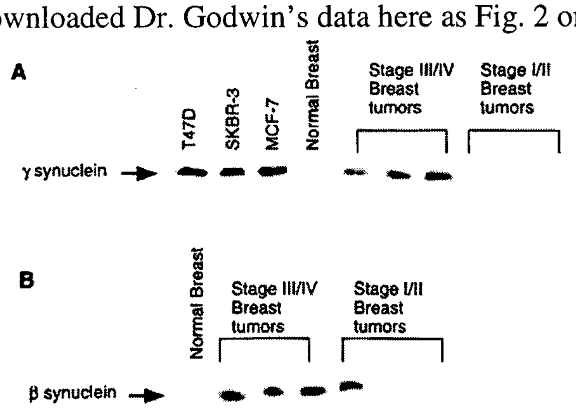


Fig. 2. Expression of γ and β synuclein in breast (Cancer 88: 2154-2163, 2000). Tissue extracts were prepared and screened by Western blotting. (A) the blot was probed with the anti-γ synuclein antibody. (B) the blot was probed with the anti-β synuclein antibody. Although β synuclein was expressed in some breast carcinomas of all stages, including ductal carcinoma in situ, SNCG expression was restricted to advanced Stage III/IV breast

carcinomas; 82% (14 of 17) of the Stage III/IV breast carcinomas expressed either BCSG1, β synuclein, or both simultaneously.

As the part of original Specific Aim 1, we also like to test whether BCSG1 can provide some prognostic information on distinguishing the DCIS (ductal carcinoma *in situ*) which is not likely to become invasive carcinoma from the DCIS which is most likely to become invasive carcinoma. Although this is a quite interesting and significant hypothesis, this aim can't be finished. PI was kind of native in his early career development to propose this kind of study, which involves a large-scale clinical follow-up of breast cancer patients and such studies are beyond the scope of the grant.

B. Effects of BCSG1 overexpression of on tumor growth and metastasis. FINISHED (18)
This specific aim is finished and the data is published in Cancer Res. 59: 742-747, 1999 (see attached Paper 1).

1. Overexpression of BCSG1 in breast cancer cells led to a profound augmentation metastasis *in vivo* (**Table 1 and Fig. 2**).
2. As to the mechanism(s) for the BCSG1-induced invasion and metastasis, we demonstrated that BCSG1-induced metastasis was not associated with the alternation in MMP and TIMP activity (**Fig. 3**).
3. Mechanistically, the induced invasion and metastasis is related to BCSG1-stimulated cell motility. We provided evidences linking overexpression of BCSG1/SNCG in human breast cancer cells with increased motility and invasive activity *in vitro* (**Fig. 4 & 5**). We also demonstrated that the BCSG1-induced cell migration is independent to the serum gradient among the top and bottom chambers in the Boyden Chamber invasion assay (Fig. 5). These data suggest that the increased migration in BCSG1 transfected cells is not likely to be mediated by chemotaxis but rather by cellular intrinsic high motile features.

Task 2: Regulation of apoptosis by BCSG1 (Not finished)

Task 3: Regulation of BCSG1 expression. FINISHED (19)
This specific aim is finished and the data is published in Breast Cancer Research and Treatment 62: 99-107, 2000 (see attached Paper 2).

1. Expression of BCSG1 mRNA in H3922 human breast cancer cells was significantly decreased by treating cells with the cytokine OM who has a growth-inhibitory effect on these cells. A decrease in BCSG1 mRNA level can be detected as early as 30 min after OM addition. By 4 h OM treatment, the level of BCSG1 mRNA was decreased to 70% of control, and by 24 h the mRNA was below detectable level (Fig. 1).
2. Western blot analysis further demonstrated the suppression of BCSG1 protein expression by OM. The level of SNCG protein in H3922 cells was reduced more than 90% by OM after 2 days (Fig. 3).
3. OM-induced down-regulation of BCSG1 mRNA occurred mainly at the transcriptional level (Fig. 4).
4. Examination of cell growth under anchorage-dependent and anchorage-independent conditions demonstrates that over expression of BCSG1 gene significantly stimulated the growth of MCF-7 cells both in monolayer culture and in soft agar. These data together suggest that BCSG1 may be one of the contributing factors that promote the uncontrolled growth of malignant **mammary** cells (Fig. 5).

III. SUMMARY AND SIGNIICANCES OF THE DATA

The metastatic potential makes breast cancer an unpredictable and incurable disease once it has metastasized to regional or distant sites. Studies linked to the discovery of new metastasis-related markers or prognostic factors will help predict recurrences, design a appropriate treatment strategy, and provide a new target for intervening metastasis.

1. We have recently identified and cloned a putative breast cancer specific gene, BCSG1, also named as SNCG. We have demonstrated that expression of BCSG1 correlate with clinical aggressiveness and may indicate breast cancer malignant progression leading to metastasis. Expression of BCSG1 in breast carcinoma is stage specific: while BCSG1 expression is not detectable in normal or benign lesions and lower percentage of BCSG1 is detected in Stage I/II breast carcinomas, but up to 70% of stage III/IV breast carcinomas express BCSG1. We also provided evidences linking overexpression of BCSG1 in human breast cancer cells with increased migratory motility and invasive activity *in vitro* and a profound augmentation of metastasis *in vivo.* These data suggest that expression of BCSG1 correlates with breast cancer progression. Therefore, analysis of BCSG1 expression may be useful in staging breast carcinomas, or predicting clinical outcomes; for example, a women whose breast tumor tests positive for BCSG1 expression is likely to have a more aggressive and invasive tumor than a woman whose breast tumor does not express BCSG1. Thus, the BCSG1 positive woman's disease should perhaps be treated more aggressively. The notion that the SNCG overexpression may indicate and facilitate breast cancer malignant progression from a benign breast or *in situ* carcinoma to a highly infiltrating carcinoma warrants further investigation.
2. Because synucleins, the neural protein mainly expressed in brain and localized to presynaptic terminals, play a critical role in neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease, elucidation of the reasons for BCSG1/SNCG overexpression in infiltrating breast cancer and BCSG1/SNCG-induced metastasis may shed some light on the pathogenesis of not only breast cancer progression but also neurodegenerative disorders.

IV. TRAINING

This is PI's first independent grant. The proposed studies of the current grant application includes a variety of different aims and experiments ranging from basic molecular biology, cell biology, *in vivo* orthotopic nude mice model for tumor growth and metastasis, and a clinical oriented study on screening clinical human breast specimens. This is the first time that PI has a chance to independently carry out a very challenge, yet ambitious, multi display project. During the last year, PI has gained a lot of experience on animal model and *in vivo* analysis of tumor metastasis. The success on the current grant proposal will encourage and facilitate PI's future career development as an independent clinically oriented breast cancer investigator. Currently, PI is intended to develop a BCSG1 transgenic mice model under the control of mammary specific promoter MMTV. This transgenic model will be used to evaluated 1) the effect of BCSG1 overexpression on mammary development and induction of mammary tumor in MMTV/BCSG1 transgenic mice; and 2) the role of overexpression of BCSG1 in breast cancer progression in MMTV/BCSG1 transgenic mice. Hopefully, with the successful development of MMTV/BCSG1 transgenic mice model, PI will be ready for a RO1 grant submission.

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[CANCER RESEARCH 59, 742–747, February 1, 1999]

Stimulation of Breast Cancer Invasion and Metastasis by Synuclein γ[1]

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ABSTRACT

We recently identified and cloned novel breast cancer-specific gene *BCSG1* by direct differential cDNA sequencing. BCSG1 has a great sequence homology with the Alzheimer's disease-related neural protein synuclein (SNC); thus, it was also named SNC-γ. Overexpression of SNC-γ in breast cancer cells leads to a significant increase in motility and invasiveness *in vitro* and a profound augmentation of metastasis *in vivo*. Our data suggest that this member of the neural protein SNCs might have important functions outside the central nervous system and may play a role in breast cancer progression.

INTRODUCTION

If sufficiently characterized, the identification of quantitative changes in gene expression that occur in the malignant mammary gland may yield novel molecular markers that may be useful in understanding breast cancer development and progression (1). Within this context, we have previously reported the isolation of differentially expressed genes in cDNA libraries from normal breast tissue and infiltrating breast cancer using the expressed sequence tag-based differential cDNA sequencing approach (2, 3). Of the many putative differentially expressed genes (2, 3), *BCSG1*, which was identified as a group of expressed sequence tags specifically expressed in the mammary gland relative to other organs and abundantly expressed in a breast cancer cDNA library but scarcely seen in a normal breast cDNA library, was identified as a putative breast cancer-specific gene (2).

Interestingly, BCSG1 revealed no homology to any other known growth factors or oncogenes; however, BCSG1 revealed extensive sequence homology to the AD[3]-related neural proteins called SNCs that are expressed mainly in the brain and localized to presynaptic terminals (4–7). The pathological hallmark of AD is amyloid deposition in neurotic plaques and blood vessels (8). Two major intrinsic constituents of amyloid are a 39–43-amino acid peptide named the Aβ component (8) and the recently identified non-Aβ component (4). The non-Aβ component of the AD precursor was cloned from a human brain library (4) and named SNCA because it shares a 95% sequence homology with rat SNC. Recently, a second SNC named SNCB was cloned from human brain, and it has a 61% sequence identity with SNCA (6). The previously identified BCSG1, which is also highly expressed in the brain (2), has a 54 and 56% sequence identity with SNCA and SNCB, respectively, and has been renamed SNCG (9). Thus, the previously unrecognized homology between these proteins defines a family of human brain SNCs that currently has three members. Although SNCs are abundant proteins expressed in presynaptic terminals and are strongly associated with amyloid plaque in AD and Lewy body in PD (10), their functions have not yet been defined. SNCA aggregation may be important in the etiology and pathogenesis of neurodegenerative disorders such as AD and PD (10). During its identification as a breast cancer-specific gene, we previously demonstrated stage-specific SNCG expression as follows: (*a*) SNCG was undetectable in normal or benign breast lesions; (*b*) SNCG showed partial expression in ductal carcinoma *in situ*; and (*c*) SNCG was expressed at an extremely high level in advanced infiltrating breast cancer. The effects of SNCG on breast cancer growth and metastasis were investigated in the current studies.

MATERIALS AND METHODS

Transfection. Full-length SNCG cDNA was inserted into a pCI-neo mammalian expression vector, and the resulting vector was transfected into MDA-MB-435 cells as described previously (3, 11).

Preparation of CM. All of the clones were maintained in subconfluent monolayers with 10% FCS. The medium was discarded, and the monolayers were washed twice with PBS. The monolayers were cultured in the absence of serum in DMEM supplemented with transferrin (1 mg/liter), fibronectin (1 mg/liter), and trace elements (Biofluids, Rockville, MD). After 24 h, the serum-free medium was discarded, and the cells were replenished with the fresh serum-free medium. The CM were collected 30 h later. Media were then centrifuged at 1,200 $\times$ *g*, and the supernatants were saved and concentrated approximately 5-fold using an Amicon hollow fiber concentrator with a M_r 10,000 cutoff at 4°C. The protein concentrations of CM were determined and normalized.

MMP Activity. The MMP enzymatic activity of the CM was assayed using a quenched fluorescent substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (Bachem) as described previously (12). The CM were pretreated with APMA for activation (13).

***In Vitro* Invasion and Motility Assay.** As described previously (11), cell invasion and motility were analyzed in a modified Boyden chamber assay using 8-μm polycarbonate membranes coated with 4 mg/ml growth factor-reduced Matrigel.

Tumor Growth in Athymic Nude Mice. A tumorigenic assay was performed in nude mice as described previously (3, 11). Briefly, approximately 0.4×10^6 cells (0.15 ml) were injected into a 5–6-week old female athymic nude mouse (Frederick Cancer Research and Development Center, Frederick, MD). Each animal received two injections, one on each side, in the mammary fat pads between the first and second nipples. Tumor size was determined at weekly intervals by three-dimensional measurements (in millimeters) using a caliper. Only measurable tumors were used to calculate the mean tumor volume for each tumor cell clone at each time point. Animals were sacrificed 32–40 days after injection, when the largest tumors reached about 15 mm in diameter.

Assessment of Regional Lymph Node and Lung Metastasis. As described previously (11), the axillary lymph nodes and lungs of sacrificed animals were excised, weighed, fixed in formalin, embedded in paraffin, and stained with H&E for a microscopic examination for morphological evidence of tumor metastasis. Sections were reviewed and scored by two pathologists.

Antibody Production. The purified synthetic SNCG peptide corresponding to amino acids 101–117 (2) was conjugated and injected into New Zealand rabbits as reported previously (12). The antiserum was purified using SNCG peptide affinity chromatography.

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[3] The abbreviations used are: AD, Alzheimer's disease; APMA, *p*-aminophenylmercuric acetate; MAP, microtubule-associated protein; MMP, matrix metalloproteinase; PD, Parkinson's disease; SNC, synuclein; SNCA, SNC-α; SNCB, SNC-β; SNCG, SNC-γ; CM, conditioned media; OM, oncostatin M.

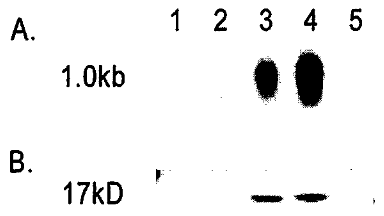

Fig. 1. Transfection of SNCG to MDA-MB-435 cells. *A*, Northern blot. Each lane contained 30 μg of total RNA. *B*, Western blot with an affinity-purified specific SNCG peptide polyclonal antibody. Each lane contained 20 μg of protein. *Lane 1*, neo-435-1; *Lane 2*, SNCG-435-2; *Lane 3*, SNCG-435-1; *Lane 4*, SNCG-435-3; *Lane 5*, neo-435-2.

RESULTS AND DISCUSSION

Transfection of SNCG into MDA-MB-435 Human Breast Cancer Cells. To determine the effects of SNCG on invasion/metastasis, we selected MDA-MB-435 human breast cancer cells as recipients for SNCG-mediated gene transfection due to their lack of detectable SNCG transcript (2) and their highly tumorigenic and aggressive phenotype in nude mice (11). Cells were transfected with a plasmid vector containing a neomycin resistance gene (neo clones) or with the same vector containing full-length SNCG cDNA (SNCG clones). MDA-MB-435 clones expressing SNCG were designated as SNCG-435 clones, and the control neo-transfected cells were designated as neo-435 clones. Fig. 1 shows the Northern blot and Western blot analyses of SNCG expression in selected clones. All selected SNCG-435 clones expressed SNCG mRNA transcripts and proteins. In contrast, none of the neo-435 clones produced any detectable SNCG transcripts and proteins. No changes in morphology were observed in these clones. Based on the level of SNCG expression, we selected SNCG-435-1, SNCG-435-3, neo-435-1, and neo-435-2 clones for the subsequent studies.

***In Vitro* Growth of SNCG-transfected Cells.** To determine whether SNCG overexpression affects the growth of MDA-MB-435 cells, cells from exponentially growing cultures of different MDA-MB-435 clones were seeded in triplicate at 3000 cells/well (24-well plate) in 1 ml of DMEM-5% serum. The growth rates of SNCG-positive SNCG-435-1 and SNCG-435-3 cells were compared with those of SNCG-negative neo-435-1 and neo-435-2 cells in a monolayer culture. No significant differences in growth rate were observed among SNCG-positive and SNCG-negative cells (data not shown).

Metastasis in the Orthotopic Nude Mice Model. Because SNCG was highly expressed in the infiltrating breast cancer cells relative to benign or noninvasive *in situ* carcinomas (2), we were interested in studying whether SNCG is an instigator of metastasis or merely a correlative product during breast cancer progression. The effect of SNCG expression on metastasis was assayed in an *in vivo* orthotopic (mammary fat pad) nude mouse model. Two independent experiments were done to confirm reproducibility, and the data from these experiments are summarized in Table 1. After a lag phase of 10 days, mice given implants of both SNCG-positive and SNCG-negative cells developed tumors. There was no difference in tumor incidence between neo-435 and SNCG-435 clones. Starting at about 20 days after inoculation, tumor necrosis was observed in tumors derived from SNCG-435-1 and SNCG-435-3 cells. Neo-435-1 and neo-435-2 cells also developed some tumor necrosis, but with less intensity. Consistent with the similar *in vitro* growth rates, there was no significant difference in primary tumor size between the neo-435 and SNCG-435 clones at 40 days after injection.

To study tumor dissemination, axillary lymph nodes and lungs were examined physically at autopsy and then subjected to microscopic examination for morphological evidence of tumor cells by light microscopy on H&E-stained paraffin sections. For the axillary lymph node, the average weight was 15 mg for neo-435 mice and 44 mg for SNCG-435 mice. The increased lymph node weight reflects the invaded breast tumors. Representative H&E-stained sections for neo-435 and SNCG-435 lymph nodes are shown in Fig. 2, *A* and *B*. Microscopic examination indicated that SNCG-435-1 and SNCG-435-3 mice showed a significantly higher average lymph node positivity (64 and 77%) compared to that (27%) of SNCG-negative neo-435-1 and neo-435-2 mice (Table 1). For lung metastases, the numbers of visible nodules on the surface of the lungs increased dramatically from an average of 1 for neo-435 mice to an average of 23 for SNCG-435 mice (Table 1). The representative lungs were shown in Fig. 2*C*. When these lungs were examined microscopically, large numbers of micrometastases were observed in SNCG-435 mice; the lungs of neo-435 mice had significantly fewer micrometastases (data not shown). Representative H&E-stained sections for neo-435 and SNCG-435 lungs are shown in Fig. 2, *D–G*. To our knowledge, human breast cancer cells usually do not form such a profound regional and metastatic tumor dissemination (visible lung nodules) in the spontaneous mammary fat pad nude mouse model. This dramatic SNCG-stimulated metastasis suggests a role for SNCG as a key positive regulator for breast cancer invasion and metastasis. The overexpression of SNCG in malignant infiltrating breast epithelial cells compared to the low expression level in noninvasive *in situ* carcinoma (2) suggests that SNCG expression is a meaningful marker for breast cancer malignant progression and may signal the more

Table 1 *Effects of SNCG on tumor incidence, tumor size, and axillary lymph node and lung metastasis*

Cells (400,000) were injected at day 1 into the mammary fat pads, and tumor volumes and lymph node and lung micrometastases were determined. Lymph node metastases were measured by microscopic examination for morphological evidence of tumor cells on the fixed axillary lymph nodes. Lung metastases were measured by the presence of visible tumor nodules on the surface of the lung. Volumes are expressed as the means ± SEs (number of tumors assayed). Experiment 1 had a total of 16 injections for eight mice in each group, and the mice were killed 42 days after injection. For experiment 2, there was a total of 10 injections for five mice in each group, and the mice were killed 38 days after injection. Statistical comparisons for SNCG-positive clones and SNCG-negative clones showed that there was no significant difference in the mean tumor sizes between pooled SNCG-positive and pooled SNCG-negative tumors. The lymph node positivity of pooled SNCG-435-1 tumors *versus* combined pooled SNCG-negative neo-435-1 and neo-435-2 tumors was $P < 0.039$ and $P < 0.029$ for pooled SNCG-435-3 tumors *versus* SNCG-negative tumors. Statistical comparison of primary tumors was analyzed by Student's t test. A χ^2 test was used for a statistical analysis of lymph node metastasis.

			Tumor incidence	Lymph node	Lymph node	Lung metastasis
Experiment	Clones	Volume (cm^3) of the primary tumor	Tumor total (%)	Average weight (mg)	No. positive/total no.	No. of nodules
1	neo-435-1	1.74 ± 0.52	16/16 (100)	14	3/16 (19)	0
	neo-435-2	1.9 ± 0.31	14/16 (88)	18	4/15 (27)	2
	SNCG-435-1	1.45 ± 0.37	15/16 (94)	43	10/15 (67)	19
	SNCG-435-3	1.78 ± 0.31	16/16 (100)	50	12/16 (75)	31
2	neo-435-1	1.35 ± 0.39	9/10 (90)	12	3/10 (30)	1
	neo-435-2	1.69 ± 0.44	10/10 (100)	15	3/9 (33)	1
	SNCG-435-1	1.73 ± 0.45	10/10 (100)	45	6/10 (60)	24
	SNCG-435-3	1.49 ± 0.34	10/10 (100)	39	7/9 (78)	17

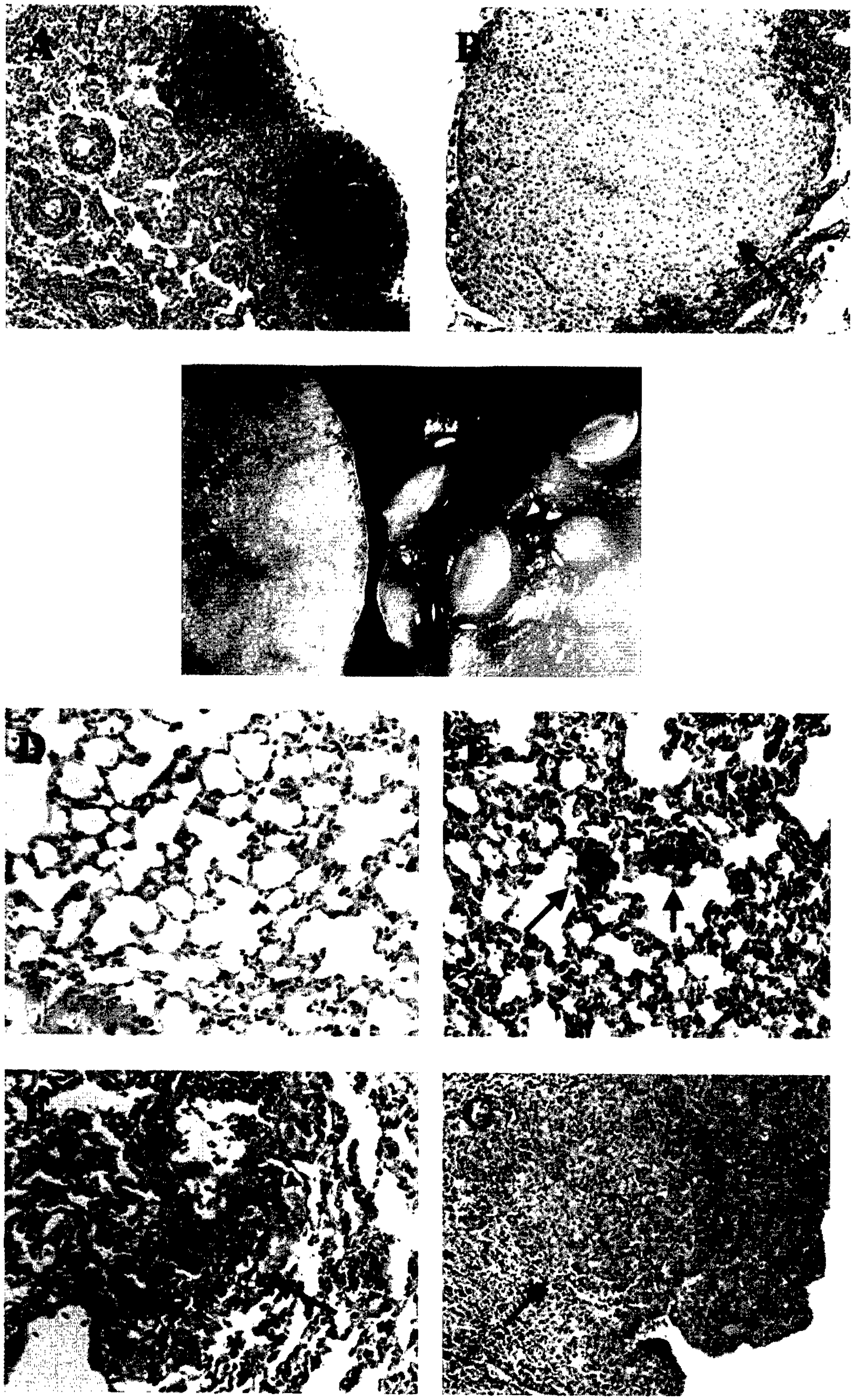

Fig. 2. Axillary lymph nodes and lung metastasis from neo-435 mice and SNCG-435 mice. The mice were sacrificed at day 40 after cell injection. Lymph nodes and lungs were isolated, and some were subjected to H&E staining. Representative axillary lymph nodes from a neo-435-1 mouse (*A*) and a SNCG-435-3 mouse (*B*) are shown. *Arrow*, an invasive breast tumor that mainly occupied the lymph node in a SNCG-435-3 mouse. *A* and *B*, ×10. *C*, representative lung metastases from mice injected with SNCG-positive and SNCG-negative cells. The *left lung* was from a neo-435-1 mouse, and the *right lung* was from a SNCG-435-3 mouse. The metastatic tumors only reflect the nodules on the surface of the lungs (×2.5). *D–G*, microscopic examination of representative lung metastases in H&E-stained sections. *D*, a lung without metastases from a neo-435-1 mouse. *E*, a lung with micrometastases from a neo-435-2 mouse. *F*, a lung with a small breast tumor nodule from a SNCG-435-1 mouse. *G*, a lung with a large breast tumor nodule from a SNCG-435-3 mouse. *Arrows*, breast tumors or cancer cells. *D–G*, ×20.

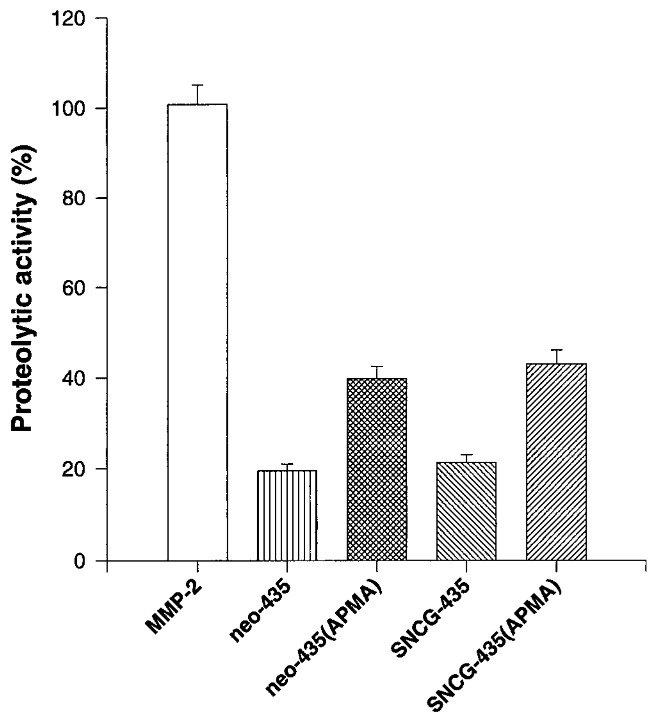

Fig. 3. Analysis of the MMP activities of SNCG-positive and SNCG-negative cells. The pooled CM from SNCG-negative neo-435-1 and neo-435-2 cells and SNCG-positive SNCG-435-1 and SNCG-435-3 cells were collected, concentrated 5-fold, normalized for protein concentrations, and subjected to MMP activity analysis. Recombinant AMPA-activated MMP-2 (80 ng) was used as a positive control. All values were normalized to the enzymatic activity of the recombinant MMP-2, which was taken as 100%. The numbers represent the means ± SD of three tests.

advanced invasive/metastatic phenotype of human breast cancer. In this regard, the up-regulation of SNCG expression may facilitate breast cancer progression leading to metastasis.

MMP Activity. In an effort to investigate the molecular mechanisms underlying SNCG-induced metastasis, we studied several invasion-related factors, including MMP and cell motility. The amyloid protein has recently been demonstrated to be a strong stimulator of MMP-2 and MMP-9 expression in astrocytes (14). It is well established that the overproduction and unrestrained activity of MMPs, particularly MMP-2 and MMP-9, are linked to the malignant conversion of a variety of different tumor cells (15–22) including mammary tumors (18–22). It is interesting to test whether SNCG, an amyloid-related protein, stimulates MMP-2 and MMP-9 expression in breast cancer cells and leads to the more metastatic phenotype. We investigated whether SNCG overexpression would increase MMP activity in MDA-MB-435 cells. In this regard, the pooled CM from two SNCG-negative cells and the pooled CM from two SNCG-positive cells were concentrated and subjected to a MMP enzymatic assay. As shown in Fig. 3, no significant differences in the basal levels of proteolytic activities were observed between neo-435 and SNCG-435 clones. Mammalian MMPs are usually secreted as latent proenzymes (zymogen) and require activation for their enzymatic activity. The incubation of CM with the MMP activator organomercurial compound APMA resulted in an approximately 2-fold increase in proteolytic activity for the CM from both neo-435 and SNCG-435 clones. However, no significant difference in APMA-activated MMP activities was observed between neo-435 and SNCG-435 clones. Because the measured enzymatic activity represents the net MMP activity, reflecting the balance between activated MMPs and the tissue inhibitors of metalloproteinase, our data suggest that SNCG-induced metastasis may not be mediated by the regulation of MMP and tissue inhibitors of metalloproteinase.

Stimulation of Invasiveness and Motility of MDA-MB-435 Cells by SNCG. We used an *in vitro* reconstituted basement membrane (Matrigel) invasion assay to determine the effect of SNCG on cell invasion. All three SNCG-negative cells (parental MDA-MB-435, neo-435-1, and neo-435-2) were moderately invasive. At the end of a 48-h incubation, an average of approximately 250 SNCG-negative cells had crossed the Matrigel barrier. A significant stimulation of invasiveness was noted in two SNCG-positive clones, with a 3-fold increase for SNCG-435-1 cells and a 4.3-fold increase for SNCG-435-3 cells (Fig. 4*A*). We also investigated the effect of SNCG on cell migration without Matrigel. A similar SNCG-stimulated pattern of migration was observed. At the end of a 24-h incubation, SNCG-435-1 cells migrated 4-fold, and SNCG-435-3 cells migrated 4.2-fold over that of average SNCG-negative cells (Fig. 4*B*). The similar magnitude of the invasion-stimulating and migration-stimulating activity of SNCG suggests that the increased invasion in SNCG clones may be mediated by an alteration of cell motility. To determine whether the increased cell motility is mediated by chemotaxis due to the different concentrations of serum or chemoattractants in the top and bottom chambers, we compared the migration of SNCG-435-3 and neo-435-1 cells under three different culture conditions: (*a*) serum-free conditions; (*b*) serum with gradient; and (*c*) serum without gradient. As shown in Fig. 5, although the migration was relatively

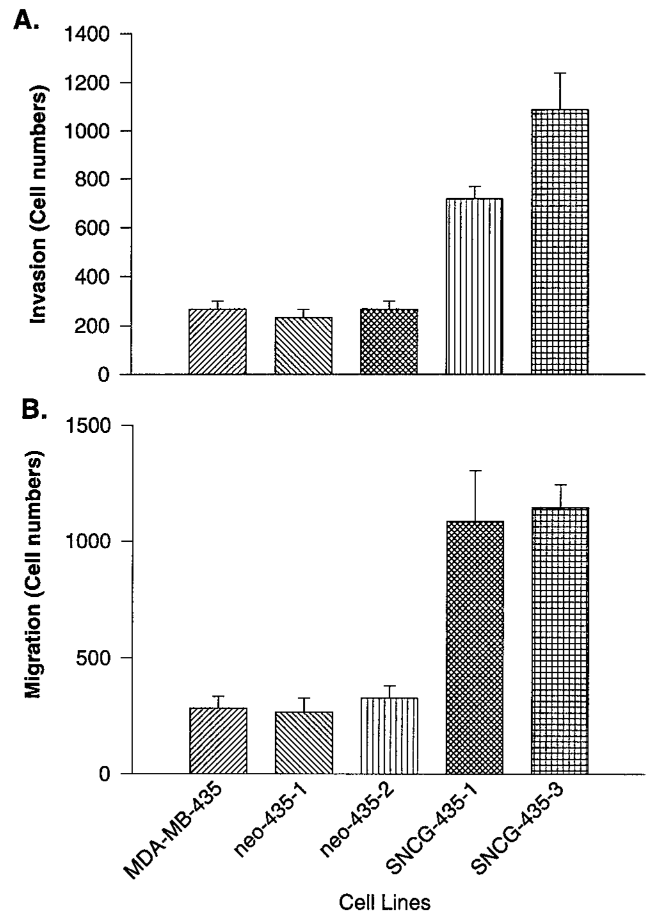

Fig. 4. Stimulation of invasiveness and migration of MDA-MB-435 cells by SNCG. Cells were seeded at a density of 30,000 cells/ml/well on 8-μm polycarbonate membranes coated with (*A*) or without (*B*) 4 mg/ml growth factor-reduced Matrigel. The top chamber contained 5% FCS, and the bottom chamber contained 10% FCS. *A*, after incubation in a humidified incubator with 5% CO_2 at 37°C for 48 h, the medium and cells were removed from the bottom chambers and counted using a microscope. *B*, cells were cultured under the same conditions as described in *A*. The number of cells that migrated was counted after a 24-h incubation. All values were expressed as the number of invaded cells. The numbers represent the means ± SD of three cultures.

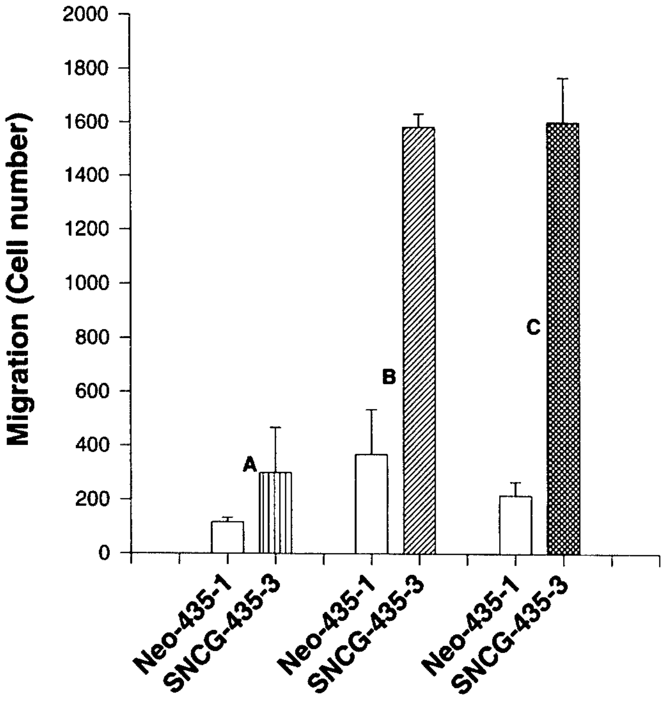

Fig. 5. Comparison of the cell migration of SNCG-435-3 and neo-435-1 cells under different conditions. Cells were cultured on noncoated membrane at a density of 30,000 cells/ml/well. The cells that migrated were harvested at 32 h after incubation. *A*, 0% serum in both the top and bottom chambers. *B*, 2% serum in both the top and bottom chambers. *C*, 2% serum in the top chamber and 10% serum in the bottom chamber. All values were expressed as the number of invaded cells. The numbers represent the means ± SD of triplet wells.

low under serum-free conditions, there was a 2.8-fold increase in migration in SNCG-435-3 cells compared with that in neo-435-1 cells. When 2% serum was added in the top chamber, the migration of both SNCG-positive and -negative cells increased significantly. However, the migration of SNCG-435-3 cells was not affected by the serum gradient. Approximately 1600 of the SNCG-435-3 cells that migrated into the bottom chamber contained either 2% serum or 10% serum. These data suggest that the increased migration in SNCG-positive cells is not likely to be mediated by chemotaxis but rather by high motility features intrinsic to the cells.

Many breast tumors go through a series of events from the time of initial detection to the formation of the lethal invasive and metastatic stage. According to the three-step hypothesis of invasion (23), cell adhesion, local proteolysis, and subsequent migration or motility are key steps in the traversal of the basement membrane and connective tissue. In this study, we provide evidence linking the overexpression of neural protein SNCG, a previously identified breast cancer-specific gene (2), in human breast cancer cells with increased motility and invasive activity *in vitro* and a profound augmentation of metastasis *in vivo*.

SNC proteins have a structural resemblance to apolipoproteins but are abundant in the neuronal cytosol and are present in enriched amounts at presynaptic terminals (9). SNCs have been specifically implicated in two diseases: AD and PD. In AD patients, a peptide derived from SNCA forms an intrinsic component of plaque amyloid (9). In PD patients, a SNCA allele is genetically linked to several independent familial cases, and the protein appears to accumulate in Lewy bodies (9). The general significance of the involvement of neural protein SNCG in cancer metastasis is unknown. Recently, SNCA and SNCB were identified as two abundant proteins through their reactivity with a monoclonal antibody recognizing MAP-τ (6) on immunoblots. In eukaryotic cells, microtubules, actin, and intermediate filaments interact to form the cytoskeletal network involved in the determination of cell architecture, mitosis, differentiation, and motility (24). Cytoskeletal organization and dynamics depend on protein self-associations and interactions with regulatory elements such as MAPs. There is increasing evidence that MAPs, including MAP-τ, play a critical role in inducing microtubule assembly and controlling the dynamic instability of microtubules, thus controlling the state of their assembly and organization in cells (reviewed in Ref. 24). SNCG may interact with MAPs and regulate the cytoskeletal organization and dynamics, leading to increased motility. Nevertheless, our data indicate that the increased expression of SNCG correlates with breast cancer progression (2) and leads to a more malignant metastatic phenotype. We also demonstrated that SNCG expression in breast cancer cells was subjected to cytokine regulation and dramatically suppressed by the tumor growth inhibitor OM, and that this OM-induced transcriptional suppression of the SNCG gene was associated with OM-mediated growth inhibition.[4] OM is an antitumor cytokine produced mainly by activated T cells and macrophages (25), and its growth-suppressing activity has been well studied in breast cancer cells (26–28). One characteristic of the host response to tumor progression is the infiltration of tumors by macrophages and T lymphocytes. The production of tumor-suppressing cytokines in a timely and locally (*in situ*) released fashion may represent an important function of the host defense system in suppressing tumor progression. From this prospective view, the dramatic suppression of SNCG expression in malignant breast cells by OM may represent the host-mediated tumor suppression leading to the inhibition of breast cancer progression.

This is the first report indicating the potential involvement of SNC in a non-neural disease. An elucidation of the reasons for SNCG overexpression in infiltrating breast cancer and SNCG-induced metastasis may shed some light on the pathogenesis of breast cancer progression as well as neurodegenerative disorders.

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Transcriptional suppression of synuclein γ (SNCG) expression in human breast cancer cells by the growth inhibitory cytokine oncostatin M

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Key words: breast cancer cell growth, gene transcription, oncostatin M, synuclein γ

Summary

Previously, we have shown that synuclein γ (SNCG), a member of the brain protein synuclein family, is highly expressed in human infiltrating breast carcinomas but not expressed in normal or benign breast tissues. The SNCG mRNA was also detected in several human breast cancer cell lines with the highest expression found in H3922, a cell line derived from an infiltrating ductal carcinoma. In this study, we show that expression of SNCG mRNA in H3922 cells is significantly decreased by treating cells with the cytokine oncostatin M (OM) who has a growth-inhibitory effect on these cells. A decrease in SNCG mRNA level can be detected as early as 30 min after OM addition. By 4 h OM treatment, the level of SNCG mRNA was decreased to 70% of control, and by 24 h the mRNA was below detectable level. Western blot analysis further demonstrated the suppression of SNCG protein expression by OM. The level of SNCG protein in H3922 cells was reduced more than 90% by OM after 2 days. Since OM- induced growth inhibition occurs after 3–4 days, the down-regulation of SNCG expression appears to proceed the effect of OM on cell growth. Additional experiments to measure the transcriptional rates of SNCG indicate that the observed OM-induced down-regulation of SNCG mRNA occurs mainly at the transcriptional level. In an attempt to examine the role of SNCG gene in the proliferation of breast cancer cells, SNCG cDNA was stably transfected into MCF-7 cells that do not express endogenous *SNCG* gene. Examination of cell growth under anchorage-dependent and anchorage-independent conditions demonstrates that over expression of *SNCG* gene significantly stimulated the growth of MCF-7 cells both in monolayer culture and in soft agar. These data together suggest that SNCG may play a role in cell proliferation.

Introduction

Breast cancer development and progression is accompanied by multiple genetic changes that lead to qualitative and quantitative alterations in individual gene expression. Consequently, the altered levels of these gene products and their cellular functions will disturb the normal physiological homeostasis of the cells and result in cancer formation. Identification of genes that are over expressed or under expressed in tumors and subsequent evaluation of their biological functions will help to understand the process of malignant transformation. By utilizing a high- throughout direct-differential cDNA sequencing approach, a novel breast cancer specific gene designated *BCSG1*, was recently isolated from a human breast tumor cDNA library [1]. The *BCSG1* gene is transcripted into a 1 kb mRNA, and the open reading frame of the full length gene is predicted to encode a 127-amino acid polypeptide.

Comparison of the predicted amino acid sequence with genetic database reveals that *BCSG1* is highly homologous to Alzheimer's disease-related neural protein synucleins (AD) that are mainly expressed in brain and are localized to presynaptic terminals [2–5]. Previously two synuclein proteins have been described, synuclein α (SNCA) and synuclein β (SNCB). SNCA is the precursor of the non-Aß fragment of human AD amyloid protein [2]. Since *BCSG1* has

54% and 56% sequence identity with SNCA and SNCB, it is also highly expressed in brain [1]. BCSG1 now is renamed as synuclein γ (*SNCG*) [6–8]. Thus, presently, the human synuclein family has at least three members.

Being identified as a breast cancer specific gene, SNCG mRNA was detected in neoplastic breast epithelial cells. *In situ* hybridization analysis has demonstrated a stage-specific expression pattern of SNCG mRNA varying from virtually no detectable expression in normal or benign breast tissue to low level and partial expression in low grade *in situ* breast carcinoma to high expression in advanced infiltrating carcinomas. These findings have been recently confirmed by examination of SNCG protein expression in breast carcinomas by immunohistochemical studies [9]. These studies suggest that SNCG may play a role in breast cancer malignant progression. This implication is supported by analysis of SNCG mRNA in several breast cancer cell lines. Northern blot analysis detected a 1 kb transcript corresponding to SNCG mRNA in 2/4 human breast cancer cell lines derived from pleural effusions and 4/4 breast cancer cell lines derived from ductal infiltrating carcinomas [1].

The high level expression of *SNCG* in the malignant breast epithelial cells suggest that the expression of *SNCG* may be up-regulated in the mammary gland during the onset and progression of breast cancer. We initiated a study to investigate whether the *SNCG* gene expression can be regulated by factors that affect the growth and differentiation of breast cancer cells. Previously we demonstrated that H3922 cells, a breast cancer cell line derived from a ductal infiltrating carcinoma, express high level of SNCG mRNA, and that the cellular proliferation of H3922 cells is inhibited by cytokine oncostatin M (OM) [1, 10, 11].

OM is a 28 kDa glycoprotein produced by activated T lymphocytes and monocytes (12–14). OM is a member of the interleukin-6 (IL-6) family of cytokines, which includes IL-6, interleukin-11 (IL-11), leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), and cardiotrophin-1 (CT-1) [15–17]. As a pleiotrophic cytokine, OM elicits a number of different biological functions in different cell types. Notable among those is its ability to regulate cell growth and differentiation. OM stimulates the growth of normal fibroblasts [18], normal rabbit vascular smooth muscle cells [19], human myeloma cells [17], and AIDS-related Kapossi sarcoma cells [20, 21]. OM also has been shown to inhibit the proliferation of a number of cell lines derived from human tumors including breast carcinoma, melanoma, and lung carcinoma [10, 11, 18, 22]. In this study, we have examined the effect of OM on *SNCG* expression and the relationship between *SNCG* expression and cell proliferation.

Materials and methods

Cells and reagents

The human breast cancer cell line H3922 was developed from a ductal infiltrating breast carcinoma at the Bristol–Myers Squibb Pharmaceutical Research Institute, Seattle [10]. Cells were cultured in Iscoves Modified Dulbecco's Medium (IMDM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Human recombinant OM was expressed by Chinese hamster ovary cells and purified as previously described [23]. The other growth factors and cytokines were obtained from R&D Systems, Minneapolis, MN.

Northern blot analysis

Total cellular RNA was isolated by the method of Peppel and Baglioni [24]. Approximately 20 μg of each total RNA sample was separated on a 1% formaldehyde agarose gel. RNA was capillary transferred to a Hybond N membrane before crosslinking to the membrane. Prehybridization and hybridization steps were performed under the conditions previously described [10, 11]. The blot was hybridized at 60°C to a 0.55 kb ^{32}P-labeled human SNCG cDNA probe. The probe was labeled using 50 μCi [α-^{32}p] dCTP with random primed DNA labeling kit (Boehringer Mannheim Corp. Indianapolis, IN). The membrane was then washed three times at ambient temperature with 2× SSC, 0.1% SDS and twice at 37°C with 0.1× SSC, 0.1% SDS. The membrane was then dried and exposed to X-OMAT scientific imaging film (Kodak, Rochester, NY) for 1–3 days at −80°C. The autoradiographs were scanned by a laser densitometer (Personal Densitometer™ SI, Molecular Dynamics, sunnyvale, CA) and the TM integrated intensity of each band was analyzed with the program ImageQuaNT™ , version 4.1. Densitometric analysis of autoradiographs in these studies included various exposure times to ensure linearity of signals.

Western blot analysis of SNCG protein

H3922 cells were cultured in 60 mm culture plates in 2% FBS IMDM with or without OM. Cells were rinsed with cold PBS and lysed with 0.25 ml of lysis buffer (50 mM Tris pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 15 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM NaF, 5 μg/ml of aprotinin, 1 μg/ml of leupeptin, 1.25 μg/ml of pepstatin, 1 mM Na_3VO_4, 10 μM of okadaic acid, and 4 μM of cypermethrin). Concentration of soluble protein from total cell lysate was determined using BCA reagent with BSA as a standard (Pierce). Forty micro gram protein of total cell lysate per sample was separated on 15% SDS PAGE, transferred to nitrocellulose membranes, blotted with rabbit anti-SNCG polyclonal antibody (1: 1500 dilution) using an enhanced chemiluminescence (ECL) detection system (Amersham). The anti-SNCG antibody, generously provided by Dr. Benoit I. Giasson at University of Pennsylvania School of Medicine, has been shown previously to specifically react with *SNCG* [25]. Membranes were stripped and reblotted with anti-ßactin mAb to ensure an equal amount of protein being loaded on gel. The signals were quantitated with a BioRad Fluro-S MultiImager System.

Nuclear run-on analysis

These analyses were conducted using a procedure adapted from one that had already been described [11]. Briefly, 1.8×10^7 adherent H3922 cells were harvested with cell scrapers into a minimal volume of cold phosphate buffered saline (PBS). The cells were pelleted by low-speed centrifugation and lysed with lysis buffer (10 mM Tris–HCl, pH 7.9, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40). The nuclei were pelleted by centrifugation and the lysis procedure was repeated once. The nuclei were recovered by centrifugation a second time and resuspended at 10^8 nuclei/ml in glycerol storage buffer (50 mM Tris–HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA). The samples were immediately frozen under liquid nitrogen and stored at −80°C. The frozen nuclei were subsequently thawed and 100 μl of each sample received 100 μl 2 × reaction buffer (70% glycerol, 0.02 M Tris–HCl, pH 7.5, 0.01 M $MgCl_2$, 0.16 M KCl, 2 mM DTT, 0.2 mM EDTA, 2 mM rATP, 2 mM rCTP, 2 mM rGTP, 2.6 μCi/μl [^{32}P] rUTP). The reactions were incubated with shaking at 30°C for 30 min. Labeled nuclei were pelleted and resuspended with 100 μl DNase buffer (50% glycerol, 20 mM Tris–HCl, pH 7.9, 1 mM $MgCl_2$, 10 mg/ml RNase-free DNase I). The reactions were incubated with shaking at 30°C for 15 min. Samples were brought up to 125 μl with 7.5 μl 13.6 mg/ml proteinase K, 5 μl 10 mg/ml yeast tRNA, and 12.5 μl 10× SET buffer (5% SDS, 0.05 M EDTA, 0.01 M Tris–HCl, pH 7.4) and incubated at 42°C for 30 min. Labeled RNA transcripts were extracted by adding the following: 275 μl GCSM solution [4 M guanidinium isothiocyanate, 0.025 M sodium citrate, pH 7.0, 0.5% Sarkosyl, 0.1 M b-mercaptoethanol], 45 μl 2.0 M sodium acetate, 450 μl water-saturated phenol, and 90 μl chloroform:isoamyl alcohol (49:1). The samples were vortexed and incubated on ice for 15 min. Nuclear run-on transcripts were precipitated with isopropanol and pelleted by high speed centrifugation. Extractions and isopropanol precipitations were repeated and the samples were dissolved with 102 μl TES buffer (10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 0.1% SDS). Assays for radioactivity were conducted by liquid scintillation. Approximately 2.0×10^6 cpm of each nuclear run-on reaction was used as a probe to hybridize a Hybond N membrane (Amersham Life Sciences, Arlington Heights, IL) slot blot. Each blot received the following two plasmids: 5 μg plasmid with the human GAPDH cDNA insert, 3 μg of the 0.3 kb fragment of the *SNCG* cDNA which is the 3′ end of the cDNA. This fragment was generated by cutting the *SNCG* cDNA with the restriction endonuclease BstXI. Probing the *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) plasmid allowed normalization of the *SNCG* signals measured by densitometry. Densitometric analysis of autoradiographs in these studies included various exposure times to ensure linearity of signals.

Transfection

The full-length *SNCG* cDNA was inserted into a pCI-neo mammalian expression vector. The *SNCG* expression vector (pCI-SNCG) and the vector alone (pCI-neo) were transfected separately into MCF-7 cells as we described previously [26]. Subsequent to transfection, after G418 selection, and cloning by limiting dilution, several subdones of MCF-7 cells were obtained. These G418-resistant clones were expanded into individual cell lines. The SNCG mRNA expressions in pCI-SNCG transfected cells, but not in muck (pCI-neo) transfected cells were confirmed by northern blot and western blot analyses.

Cell proliferation assay

Exponentially growing cultures of different MCF-7 clones were detached with trypsin, and the trypsin was neutralized with DMEM with 10% serum. Cells were counted, diluted, and seeded in triplicate at 3,000 cells per well (24-well plate) in 1 ml DMEM with 5% serum. Cell growth was measured using a cell proliferation Assay Kit (Promega, Madison, WI).

Soft agar assay

The anchorage-independent growth was carried out in 35 mm^2 culture dishes. The bottom layer consists of 0.8 ml of 5% FBS/IMDM containing 0.6% agar. The top layer consists of 0.8 ml of 5% FBS/IMDM containing 0.4% agar and approximately 6000 cells. The cells were cultured in an atmosphere of 5% CO_2/95% air under saturating concentrations of humidity at 37°C. After two weeks, the number of colonies was counted by using Omnicon 3600 Image Analysis System.

Figure 1. Time-dependent suppression of SNCG mRNA expression by OM. Total RNA (20 μg/lane) was isolated from H3922 cells that were cultured in 2% FBS IMEM and treated with OM at a dose of 20 ng/ml for the indicated lengths of time. RNA samples were blotted onto a nylon membrane and hybridized to a ^{32}P-labeled 0.55 kb SNCG cDNA probe as described in 'Materials and methods'. The blot was rehybridized under the same conditions with a ^{32}P-labeled human GAPDH probe. Radioactive signals were detected by autoradiography and quantified by densitometry. The figure shown is representative of three separate kinetic studies.

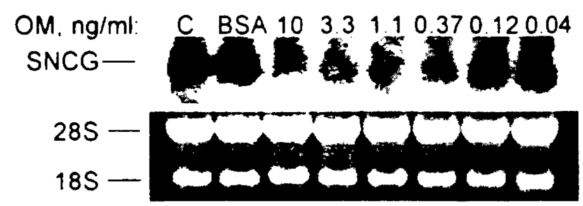

Figure 2. OM concentration-dependent effects on SNCG mRNA expression. OM was added to H3922 cells at the concentrations indicated. Total RNA was isolated after 16 h OM treatment. The figure shown is representative of three separate northern blots.

Results

Time- and concentration-dependent suppression of SNCG mRNA expression in breast cancer cells by OM

The effect of OM on SNCG mRNA expression in H3922 cells was examined by northern blot analysis. The results in Figure 1 demonstrated a time-dependent suppression of SNCG mRNA by OM. Treatment of H3922 cells with OM initiated an immediate decrease of SNCG mRNA as early as 30 min. By 4 h treatment, the level of SNCG mRNA was decreased to 70% of that in control cells and by 24 h the mRNA level was below the detectable level. The suppressive effect of OM is persistent, as SNCG mRNA was not yet detected in H3922 cells after withdrawing OM from the culture medium for two days, but the mRNA started to appear at low levels after three days (data not shown).

OM-suppressed SNCG mRNA expression was also concentration dependent. After 16 h treatment, OM at concentrations as low as 0.37 ng/ml caused significant decrease of *SNCG* expression (46% of control), whereas maximal suppressions (approximately 20–25% of control) were observed at concentrations of 1.1 ng/ml and higher (Figure 2), whereas the OM diluent (BSA 1 mg/ml in PBS, lane 2) had no effect. This concentration range is slightly lower but comparable to those for inhibition of cell growth (2–10 ng/ml) [10, 11, 22]. These data suggest that inhibition of cell growth and suppression of SNCG mRNA expression might be related.

To examine the effect of OM on SNCG protein expression, H3922 cells were cultured in the absence or the presence of OM for 2–4 days and then untreated and OM treated cells were lysed. Total cell lysate was harvested and 40 μg soluble protein from each sample was loaded on a 15% SDS gel and separated by electrophoresis, transferred to a nitrocellulose membrane, and blotted with rabbit anti-SNCG polyclonal antibody. Figure 3 shows that SNCG protein level was decreased more than 90% in OM-treated cells (lanes 3, 4) as compared to that in control cells (lanes 1, 2). In contrast to SNCG, the level of ß-actin was not altered by OM (data not shown).

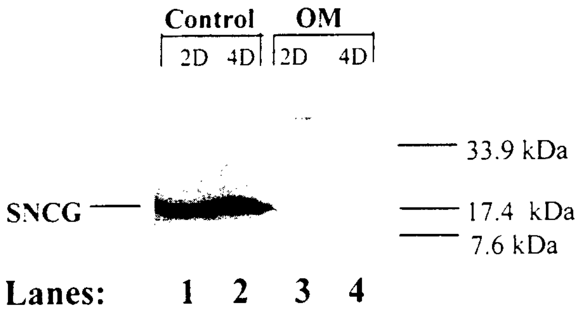

Figure 3. Western blot analysis of SNCG protein in H3922 cells. Total cell lysate was isolated from H3922 cells that were untreated or treated with OM for 2 or 4 days. Untreated control cells and OM treated cells were lysed simultaneously at the end of treatment. Forty micro grams of total cell lysate per sample was analyzed for SNCG protein expression by western blot.

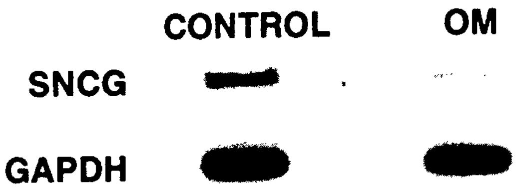

Figure 4. Nuclear run-on analysis of SNCG transcription. Two slots were blotted onto each of two nylon membrane strips. One slot received 3 μg of the 0.3 kb fragment of the SNCG cDNA, which is the 3′ end of the cDNA. The second slot was loaded with 5 μg of the GAPDH plasmid. One nylon strip was hybridized to a ^{32}P-radiolabeled nuclear run-on reaction prepared from 16 h OM-treated H3922 cells. The second was hybridized to a labeled nuclear run-on reaction prepared from untreated control cells. Equal amounts of radioactivity were used in each hybridization. Radioactive signals were detected by autoradiography and quantified by densitometric analysis. The figure shown is representative of three separate assays. Densitometric analysis of autoradiographs in these studies included various exposure times to ensure linearity of signals. No hybridization signals were detected on slots containing plasmid bluescript DNA.

Transcriptional suppression of SNCG mRNA expression by OM

To determine whether the down regulation of *SNCG* expression by OM occurs at the transcriptional or post-transcriptional level, we conducted nuclear run-on assays to measure the relative transcription rate of SNCG in control cells and in the cells treated with OM. As shown in Figure 4, treatment of H3922 cells with OM for 16 h, decreased the level of actively transcribed SNCG mRNA to 28.5% of that in untreated cells. Data were normalized by the signals observed in the GAPDH slots. The level of reduction of SNCG transcripts is comparable with the results obtained from northern blot analysis (Figure 2), suggesting a transcriptional regulatory mechanism.

Overexpression of SNCG *gene in breast cancer cells stimulates cell growth*

To further investigate the biological relevance of *SNCG* expression to the growth of breast cancer cells, we selected MCF-7 cells as a recipient cell line for *SNCG* mediated gene transfection due to its lack of endogenous SNCG mRNA expression [1]. MCF-7 cells were transfected with a plasmid vector containing a neomycin resistance gene (neo-MCF clones), or the same vector containing a full-length *SNCG* cDNA (SNCG–MCF clones). Individual clones were initially screened by *in situ* hybridization on slides with a specific *SNCG* antisense probe, and the positive clones were subjected to northern blot analysis. Figure 5A shows the northern blot analysis of *SNCG* expression in randomly selected clones. All selected SNCG–MCF clones expressed SNCG mRNA transcripts. In contrast, none of the neo-MCF clones produced any detectable SNCG mRNA. SNCG protein expression in the positive clones was confirmed subsequently by western blot analysis using anti-SNCG polyclonal antibody (Figure 5B). No changes in morphology were observed in these clones.

To determine whether *SNCG* overexpression affects the growth of the transfected cells, the growth rates of SNCG positive MCF-7 clones (SNCG–MCF-2 and SNCG–MCF-6) were compared to that of SNCG negative MCF-7 cells (neo-MCF-1 and neo-MCF-2) in both monolayer culture and soft agar. Figure 6 shows that the anchorage-dependent cell growth was significantly stimulated in SNCG transfected cells compared to neo-MCF cells to approximately 3.2-fold ($p < 0.001$ by Student's *t-test*). Similar results were obtained from soft agar assay as shown in Table 1.

To determine whether overexpression of SNCG will alter the response of MCF-7 cells to OM, the transfected SNCG-MCF cells and the untransfected parental MCF-7 cells were treated with OM for 6 days and then the cell number were counted. The results showed that the cell numbers in OM treated MCF-7 cells were decreased to 42.3% of control. In contrast, the growth of SNCG-MCF-7 cells was not affected by OM, suggesting that over expression of SNCG renders the cells resistant to OM-mediated growth inhibition.

OM-specific receptor type II mediates the down regulation of SNCG *gene expression*

The biological activities of OM can be mediated through two types of receptor complexes, the LIF/OM

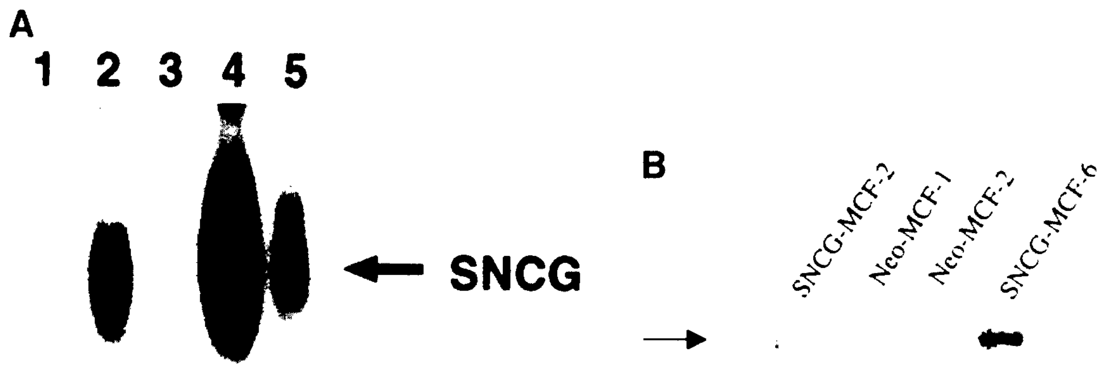

Figure 5. Detection of SNCG mRNA and protein expression in transfected MCF-7 cells. (A) Total RNA was isolated from each clones and 30 μg of total RNA was analyzed. Lane 1, neo-MCF-1; lane 2, SNCG-MCF-2; lane 3, neo-MCF-2; lane 4, SNCG-MCF-6; and lane 5, SNCG-MCF-4. (B) Total cell lysates were isolated from mock-transfected clones neo-MCF-1, neo-MCF- 2, and SNCG-transfected clones SNCG-MCF-2, SNCG-MCF-6. Fifty micro grams protein of total cell lysate per sample was analyzed for SNCG protein expression by western blot. Equal amount of soluble protein from each lane was verified by blotting with anti-β actin monoclonal antibody.

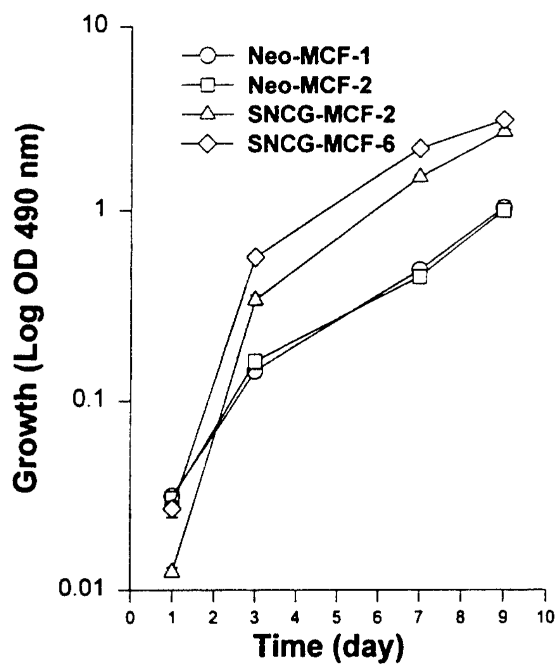

Figure 6. Cell growth assay of SNCG transfected MCF-7 cells. Cells were cultured in 5% FBS DMEM medium with a seeding density of 3000 cells per well in 24 well culture plates. Cell number was determined after culturing cells at the indicated time. The data shown are representative of five experiments in which triplicate wells were assayed for each data point.

Table 1. Comparison of the growth rate of SNCG-MCF-7 cells with neo-MCF-7 cells in a soft agar assay

Cell lines	Number of colonies
Neo-MCF-1	142 ± 38
Neo-MCF-2	185 ± 51
SNCG-MCF-2	553 ± 59
SNCG-MCF-6	334 ± 41

Cells were plated at a density of 6,000 cells/35 mm^2 culture dish in IMDM containing 5% FBS and 0.4% agar. The number of colonies was counted after 2 weeks of plating using Omnicon 3600 Image Analysis System. Colonies smaller than 60 mm were excluded.

shared receptor (type I) and OSM-specific receptor (OSMR, type II) [27–29]. Previous studies conducted in our laboratory showed that the growth-inhibitory activity of OM in the H3922 breast cancer cells is mediated through OM-specific receptor type II [10, 30]. Although the type I OM receptor that mediates the actions of LIF and OM is also expressed in these cells, LIF did not inhibit the growth of these cells, instead slightly stimulated their growth [10]. In addition, previous study showed that IL-6 and IL-11 do not affect the growth of these cells either [10]. To determine whether the effect of OM on *SNCG* gene expression is mediated through the type II OM-specific receptor, the effect of LIF, along with IL-6 and IL-11 on *SNCG* expression was compared with OM. H3922 cells were treated with individual cytokines for 24 h at a concentration of 100 ng/ml. The results of northern blot analysis show that SNCG mRNA expression was marked suppressed by OM, but not significantly suppressed by LIF, or IL-6 and IL-ll (Figure 7). These data

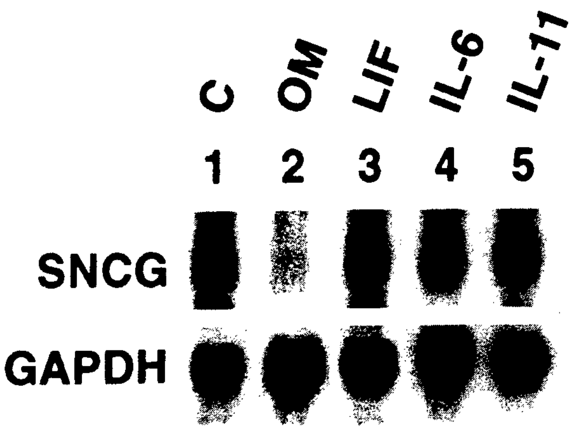

Figure 7. Comparison of effects of OM related cytokines on *SNCG* gene transcription. H3922 cells were treated for 24 h with each factors at 100 ng/ml concentration respectively. Total RNA was subsequently isolated and northern blot analysis of SNCG mRNA levels was performed.

suggest that the type II OM specific-receptor transmit OM elicited signals that lead to the repression of SNCG transcription.

Discussion

Similar to other synuclein proteins, the normal expression of *SNCG* is restricted to brain tissue [6]. Although synucleins are abundant proteins expressed in presynaptic terminals, and SNCA has been found to be a major protein component of the amyloid plaque in Alzheimer's disease and lewy body in Parkison's disease [5], their functions have not been defined yet. The finding that *SNCG* is highly expressed in advanced infiltrating breast carcinomas, but not expressed in normal breast tissue, suggest that these neural proteins might have important functions outside the central nervous system. Furthermore, the overexpression of *SNCG* in advanced breast carcinomas suggest that *SNCG* expression might be regulated by factors which play key roles in the complex regulation of the growth and progression of breast carcinoma. Indeed, in this report, we have demonstrated that SNCG is transcriptionally modulated by the growth-inhibitory cytokine oncostatin M.

We show that expression of SNCG mRNA was rapidly down regulated by OM. After 24 h treatment with OM, SNCG mRNA was below the detectable level in H3922 breast cancer cells. Interestingly, the kinetics of OM induced down regulation of SNCG was different than the kinetics of OM-induced down regulation of the *c-myc* gene in these cells. C-myc mRNA was transiently induced by OM within 1–4 h and subsequently suppressed at later times. The maximal suppression (20–30% of control) occurred after 3 days of OM treatment at which time the growth was strongly inhibited by OM [10, 11]. Thus, the suppression of *SNCG* gene proceeds the inhibition of *c-myc* gene and cell proliferation. It appears to be an early step in OM-induced cellular events that ultimately lead to cell growth arrest.

The concurrent effects of OM on cell growth and *SNCG* expression suggest that *SNCG* may play a direct role in the abnormal growth of the breast cancer cells. This hypothesis is supported by the fact that transfection of *SNCG* gene into MCF-7 cells resulted in a 3-fold increase in cell growth under both anchorage-dependent and independent conditions.

It is interesting that MCF-7 cells do not express endogenous SNCG mRNA and the cell growth is inhibited by OM. However, MCF-7 cells become resistant to OM-mediated growth inhibition when *SNCG* gene is over- and constitutively expressed. *SNCG* is likely to be one of many cellular genes that participate in the control of cell proliferation. Growth inhibition of OM involves alteration of transcription of several cellular genes including *c-myc*, *p53* [31], *SNCG*, and may be some other genes, such as *p21*, in a concert action. It is likely that down regulation of *SNCG* gene expression by OM is part of this concert process leading to the growth inhibition. In the situation of SNCG being over expressed and unregulated, it may overcome the suppressive effects of OM on other genes, such as *c-myc*, and *SNCG* becomes a dominant factor to promote cell growth over other gene products. This hypothesis will be further examined by expressing *SNCG* under an inducible expression system in MCF-7 cells. That will allow us to examine the relationship between OM-mediated growth inhibition and *SNCG* expression in a better-controlled system.

As a highly expressed gene in advanced breast carcinomas, *SNCG* could be multi-functional. Recently, we also showed that expression of *SNCG* in breast cancer cell line MDA-MB 435 cells led to a significant increase in motility and invasiveness in vitro and a profound augmentation of metastasis *in vivo* [8]. These data suggest that *SNCG* may be involved in both the growth and the metastasis of the breast cancer cells. Since the normal functions of these synuclein proteins in brain are not yet defined, elucidating the biological activities of *SNCG* and the mechanism for its abnormal expression in breast cancer will provide important clues to understand the pathogenesis of not only breast cancer progression but also neurodegenerative diseases.

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